Heat-induced maternal effects shapes avian eggshell traits and embryo development and phenotype in high incubation temperatures

Alexander Hoffman (ajh0077@auburn.edu)
Auburn University https://orcid.org/0000-0003-1224-9965
Leslie Dees
Auburn University
Haruka Wada
Auburn University

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Abstract

Populations without a sufficient rate of genetic adaptation may risk extinction in the face of rapid environmental change, however, phenotypic plasticity can facilitate their persistence. For example, mothers can prepare offspring for the thermal environment they will experience through maternal effects. In oviparous species, little is known about how variation in the timing and intensity of thermal stressors influences embryo phenotype. We demonstrate that when zebra finch mothers are exposed to a mild heat conditioning as juveniles, their embryos do not exhibit the increased water loss and shorter development times at high incubation temperatures shown by embryos from control mothers. Mothers exposed to the mild heat conditioning as juveniles and a high heat stressor as adults produced eggs with a higher density of shell pores, embryos with lower heart rates during development, and hatchlings with heavier pectoralis muscles when incubated at a high temperature. However, maternal heat exposure was only positively correlated with embryo survival at a control temperature, where embryos produced by mothers exposed to only the mild heat conditioning exhibited higher survival rates than all other groups. These results demonstrate that the thermal environment experienced by mothers can influence the physiology of their embryos and eggshell characteristics.

Introduction

Thermal biology drives geographical range limits of a species, and those that cannot rapidly adapt or migrate may experience severely diminished populations or extinction in the face of climate change (*1*). Populations with slower growth rates such as those with long generation times risk extinction before genetic adaptation to the new environment can occur (*2*). However, phenotypic plasticity (the ability of one genotype to produce multiple phenotypes) may give organisms a faster mechanism of persistence in rapidly changing climates compared to genetic adaptation. The environmental conditions experienced by parents can also affect the phenotype of offspring without alteration of DNA. This is known as transgenerational phenotypic plasticity, or maternal effects when mediated by the mother (*3*).

Maternal effects can improve the fitness of offspring in a changing environment by adjusting their phenotype to match the expected environmental conditions (*4*). These are often called anticipatory maternal effects (*3*). However, maternal effects may not always be beneficial to offspring fitness. In some cases, these effects may increase maternal lifetime fitness while having a detrimental effect on the performance of current offspring, sometimes known as selfish maternal effects (*3, 5*). This is in large part because selection acts to maximize fitness of the mother rather than that of the individual offspring, although they are often positively correlated (*5*). In order for maternal effects to be adaptive, it is necessary that the environment experienced by the mother is heterogeneous and an accurate predictor of the environment that will be experienced by offspring. Thus, if the offspring's environment does not match the maternal conditions which induced the phenotypic change, offspring fitness can be decreased (*3*).

The magnitude of the environmental conditions or stressor can influence the strength and direction of maternal effects (*3*). This is likely because the strength of the stressor is informative of its reliability as a predictor of future conditions (*6*). If an environmental stressor experienced by the mother is chronic, it may suggest that the change in conditions will persist and also be experienced by the offspring, strongly necessitating a phenotypic change to maintain fitness (*6*). When such chronic stressors are mild, they can also induce within-generation adaptive phenotypic plasticity, sometimes known as conditioning hormesis (*7, 8*). On the other hand, an environmental stressor of short duration could be unpredictable and a poor indicator of future conditions, and less likely to induce long-term phenotypic change. For example, adult sheepshead minnows exposed to 24° C and 34° C for 30 days produced offspring with increased growth at the temperature their parents were subjected to. However, this parental effect was not seen when the parents were exposed to the temperatures for 7 days rather than 30 days (*9*). The ontogenetic timing of environmental cues also has a large effect on the induction of maternal effects (*10*). The developmental environment is considered a critical window for generating adaptive plasticity, as conditions experienced during this time can have persisting and irreversible effects on phenotype. Moreover, research has shown that early development is also a critical time-period for inducing transgenerational plasticity specifically, and in some species, it may be the only time at which such effects can be induced (*11, 12*). However, experiments that incorporate multiple exposure points and stressor magnitudes experienced by mothers is limited, though such data may be critical in determining how life-history influences the generation of maternal effects.

Although avian embryos develop outside of the mother in an egg, providing a virtually closed environment, prenatal maternal effects play an important role in many species (*13*). Avian embryos are also functionally ectotherms, and rely mostly on parental incubation for thermoregulation. Thus, incubation behavior of parents heavily affects incubation temperatures and influences development and survival of embryos (*14*). Embryos of many avian species are able to withstand low ambient temperatures for relatively prolonged periods, as parents must leave the nest to forage, leading to temporary cooling of the egg (*14, 15, 16*). In contrast, most embryos cannot withstand hyperthermic conditions for very long (*15, 16*). Rising incubation temperatures cause an increase in embryonic metabolic demands, resulting in higher oxygen consumption, water loss, and CO_2 build up, eventually leading to desiccation, respiratory or cardiac failure, and death (*17*). A high eggshell gas conductance can ameliorate some of these effects by increasing the rate by which oxygen diffuses in and CO_2 diffuses out. However, this comes with the trade-off of increasing water loss to potentially fatal levels (*17).* The rate of gas exchange for embryo metabolic demands are influenced by the pore density and thickness of the eggshell, and therefore these traits can largely impact development, survival, and the environment *in ovo.* Eggshell pore density and thickness differs across species ranges due to adaptation to varying environmental conditions such as temperature, humidity, and altitude, which is thought to occur across many generations (*18, 19*). However, if these eggshell characteristics possessed some degree of plasticity, it could in part facilitate embryonic survival when faced with rapid change in ambient temperatures in a single generation.

In this study, we used zebra finches to investigate how maternal exposure to thermal stressors of varying magnitudes and at different points in their life cycle influences their offspring's phenotypes as well as eggshell characteristics. Additionally, we examined how these changes influence embryo performance and survival at multiple incubation temperatures. To test this, we exposed juvenile female zebra finches to a mild heat conditioning (38° C) or control (22° C) temperature treatment every other day for 28 days. As adults, the finches were then exposed to either a high heat stressor (42° C) or control temperature (22° C) treatment for 3 consecutive days. These females were paired to breed with non-experimental males, and the resulting eggs laid were collected and then incubated for the duration of embryonic development at either a control temperature (37.2°C), or a high temperature (38.5°C), which has been shown to decrease hatching success in this species (*20*). We then measured several developmental and physiological parameters of offspring such as embryonic heart rate and development time, change in egg mass during incubation, and hatchling morphology. We also measured eggshell pore density and thickness to determine if these traits are subject to maternal effects, and whether this plasticity is associated with changes in offspring phenotype. The factorial design used in this study allows us to determine how offspring phenotypes were influenced by maternal thermal exposure, and the potential interactions with the incubation temperature embryos developed in. We predicted that exposure to a mild heat conditioning treatment as juveniles would induce an anticipatory maternal effect, resulting in females producing offspring that are better suited to cope with high incubation temperatures. We expected that these embryos would exhibit less water loss, lower heart rates, higher organ masses, and eggshells with increased pore density when compared to embryos from juvenile control mothers. We also predicted that maternal exposure to only an acute and high intensity thermal stressor in adulthood prior to breeding would induce a selfish maternal effect, producing offspring that have reduced performance and survival regardless of incubation temperature.

Materials And Methods

Animal diet and husbandry

For a full description of animal diet and husbandry methods see supplementary materials or Hoffman et al. 2018 (*21*).

Juvenile treatment

A total of 48 females underwent the juvenile conditioning treatment, following a protocol that has been previously established (*22*) and used in our laboratory (*21*). Females were semi-randomly assigned to either the control or mild heat treatment group, balancing within nest and hatch order. At 42-45 DPH, half of the juvenile females began the mild heat treatment and were exposed to a temperature of 38°C on every other day for 28 days as previously described (*21, 22*). This temperature of 38°C can be considered a mild heat stressor because although it is still within the thermoneutral zone for the species (~30 - 40°C), it is near the upper critical temperature (*23*). For this species, body temperature begins to increase at ambient temperatures higher than 30°C, leading to mild hyperthermia (*23*). This increase in body

temperature limits water loss by creating a thermal gradient allowing for better heat dissipation, and may initiate stress responses such as glucocorticoid secretion and heat shock protein synthesis (*24, 25*) without increasing oxidative damage (*22*). A study by Costantini et al. (2012) demonstrated that when zebra finches were exposed to this early life thermal conditioning protocol, they had increased resistance to oxidative damage when exposed to a high intensity thermal stressor later on as adults (*22*). In the wild, even some of the hottest regions in Australia (such as Alice Springs and Telfer) historically experience less than 28 days a month with temperatures above 35 °C in the summer (*26*). However, these numbers are increasing and are predicted to increase even further as a result of climate change (*27*). The other half of the juvenile females were used as a control group, and were placed into brooders in the same manner as the mild heat group, but instead set to room temperature at 21-23°C. Although this temperature is below the species thermoneutral zone, it was used as a control treatment because it is the temperature at which they, as well as previous generations were reared.

Adult treatment

At 170-180 DPH, half of the females from each juvenile stage treatment were exposed to a high heat stress treatment of 42°C for 3 hours a day, for 3 consecutive days as previously described (*21, 22*). This temperature is above the upper critical temperature of the thermoneutral zone and has been shown to cause oxidative damage in this species (*22, 23*). The remaining females from each of the juvenile stage treatment groups served as the adult-stage control group, and were exposed to room temperature (21-23°C) in the same manner as above. This 2 x 2 factorial design resulted in four maternal treatment groups that were the different combinations of juvenile and adult treatment (Control-Control, Control-Heat, Heat-Control, and Heat-Heat). The females were also weighed the day before, and on the final day of the adult treatment following the last treatment bout.

Female pairing

Females were paired with non-experimental males and on the day of pairing, females first underwent a single 3-hour bout of their respective adult-stage treatment (control or high heat) as described above. Pairs were housed in the same breeding conditions and received the same diet as previously described. A total of 43 females successfully produced eggs that underwent the incubation stage treatment (*n*: CC – 12, CH – 12, HC – 10, HH – 9).

Egg collection and incubation treatment

After pairing, nests were checked for eggs daily at approximately 1000 hrs. Once laid, eggs were removed from the nest and marked with a unique ID number using a fine tip permanent marker. The first egg laid in the clutch was replaced with a false egg made of modeling clay (Crayola Model Magic (white), Easton, PA) after removal, while any subsequent eggs removed were not replaced. The first overall egg laid was randomly assigned to an incubation treatment (a control or high incubation temperature), and following that the first laid egg of each subsequent nest was assigned a treatment group in an alternating manner to balance treatment allocation among the first eggs of the clutch. After the first egg, treatment group

assignments alternated within each clutch to balance laying order and nest of origin between incubation temperature treatments. Therefore in general, there were multiple embryos in both treatment groups for each female.

Newly laid eggs were transported to the lab where they were weighed and then placed into one of two incubator treatments, representing a control or high incubation temperature. Those assigned to the control group (*n* = 125 embryos) went into an incubator (Brinsea Octagon 20 Advance EX incubator, Titusville, FL) set to 37.2° C for the entirety of embryonic development. This temperature was chosen as it is very close to the average incubation temperature of captive zebra finches (*28*) and was found to be optimal when considering both hatching success and post-hatch survival (*20*). Individuals assigned to the high incubation temperature (*n* = 106 embryos) were placed into an incubator set to a temperature of 38.5°C, which is slightly above the range of incubation temperatures for wild and laboratory kept zebra finches (*28*), for the entire incubation period. In this species, an incubation temperature of 38.4° C can result in lower hatching success overall and in lower overall and lean body mass in male offspring during their postnatal growth period (*20, 29)* and therefore considered outside the thermal optimum. Both incubators were set to a relative humidity of 55%. Incubators were calibrated to the desired temperature using a reference thermometer placed at level with the eggs. Approximately three times per week, incubators were taken off of their rockers and allowed to sit level for at least one hour before reading the thermometer to determine temperature accuracy. The average measured temperature in control and high temperature incubators were 37.13°C ± 0.512 and 38.45°C ± 0.419 (mean ± standard deviation), respectively.

Heart rate measurements

Embryonic heart rates were measured using the Buddy® digital heart rate monitor (Avian Biotech, Vetronic Services, Abbotskerswell, Devon, UK). This is a non-invasive method which quantifies heart rate by the amount of infrared light absorbed by the pulsing embryonic blood. The Buddy® monitor has been used in a variety of studies on oviparous embryos, often examining the effects of incubation temperature on reptilian embryos (*30, 31, 32*). It has also been used in thermal manipulation studies in poultry and turkey embryos as well as studies on other avian species using a variety of stimuli (*see 33*). More recently, a thorough investigation using the Buddy® system has been done on wild zebra finch embryos (*33*).

Heart rate measurements were recorded repeatedly at two different time points during an individual's embryonic development, between 1400 and 1700 during the day to account for any circadian variation. The first measurement was taken at embryonic day (ED) 4, which is at ~28.9% of development for embryos in the control temperature incubator and ~29.4% of development in the high temperature incubator. The second measurements were taken at ED 11 for embryos in the control incubator and at ED 10 for embryos in the high incubator, which is ~79.6% and ~73.5% of development respectively.

When measuring heart rates, eggs were individually removed and placed horizontally (pointed tip facing digital display) on the measuring platform of the monitor before closing the lid. A timer was started as

soon as the egg was removed from the incubator, and the amount of time (s) that the embryo had been out of the incubator at each heart rate (bpm) measurement was recorded. This value was used in the analysis to account for the decrease in heart rate when eggs were removed from the incubator and began to cool. Heart rates were recorded every 10 seconds after the first recording until the egg had been outside of the incubator for ~70 seconds. If reliable heart rates could not be picked up by the monitor by the time the egg had been out of the incubator for ~40 seconds, the egg was flipped 180° on the platform. Measurements were considered reliable when the output values were consistent and continuous, with the "heart icon" on the digital display blinking. Eggs did not remain inside the monitor for longer than ~90 seconds. For day 4 measurements, eggs were immediately placed back into their respective incubator after heart rates had been recorded. For day 10/11 measurements, each egg was weighed after recording the heart rate before being returned to incubators. Egg did not remain outside of the incubators for longer than ~240 seconds total while recording heart rate measurements and egg mass. The heart rate measurement taken between the 50 and 60 second mark was used in statistical analyses. The order in which eggs were removed from the incubator for measurements was also recorded to be taken into account in the statistical analysis. When no heart rate signal could be found using the monitor, eggs were candled using a flashlight to determine infertility or mortality. If an egg was found to have a crack due to handling or collisions inside the incubator, the heart rate was not measured and that individual was not used in survival analysis. When an embryo was found to be dead and had no cracks in the shell, the developmental stage (very early, early, mid, late, very late, pipping) at which mortality occurred was estimated and recorded for later use.

Organ measurements

All incubators were checked a minimum of three times daily for potential hatchlings. When a hatchling was found, it was removed from the incubator, weighed, and then euthanized via isoflurane inhalation as per approved protocol. Dissected tissues included the residual yolk sac, heart, and pectoralis muscle, and the tissues were weighed prior to freezing. After removal, all dissected tissues were then flash frozen in liquid nitrogen before being placed in a -80° C freezer, while the carcass was placed in a freezer at -20° C for later use.

Eggshell measurements

Pore density was determined using a protocol refined for songbird eggs by Stein and Badyaev (*34*) with some modification. Briefly, pieces of shell from each egg were placed in a biopsy case. The shells were then incubated in hot NaOH bath for 3 minutes to remove the membrane. Following this, the shells were rinsed in ddH2O, then dried in an incubator set to 41°C. Once dry, the thickness of the shell was measured using a micrometer (Mitutoyo, cat number 395-371-30, 0.001mm resolution) to the nearest 0.001mm. Shells were then incubated in 2.5% nitric acid for 12 seconds, followed by a rinse in ddH_2O. The shells were dried once again in an incubator set to 41°C. Once dry, the number of eggshell pores were counted using a Nikon Eclipse microscope. One researcher measured the thickness and another researcher

counted all the pores for all samples. Both researchers were blind to the treatment groups from which the eggshells came.

Statistical analyses

Statistical analysis was done using RStudio (*35*) utilizing the packages “nlme” and “coxme”, and graphs were constructed using the package “ggplot2”. Statistical significance was determined as $P \leq 0.05$, and all models included the maternal ID as a random effect to account for multiple eggs originating from the same mother. Predictors that were believed to have plausible biological effect on some aspect of the model were included regardless of significance. Assumptions of linearity of the data were tested by plotting the residuals versus fitted values. Assumptions of normality of residuals were tested via histogram plots of the data for linear models. For Cox proportional hazard (PH) models, assumptions of proportional hazards were examined using a Schoenfeld’s test. For linear models, regression coefficients were used for estimates of mean change in response variables, while hazard ratios were used for estimates in Cox PH models. Estimates of uncertainty were represented via 95% confidence intervals, calculated using the functions “intervals” and “confint” for linear regressions and Cox PH models respectively. For details on the specific statistical models used for data analysis of each variable reported, see supplementary materials.

Results And Discussion

For avian embryos, the demand for oxygen generally increases as temperatures rise, leading to an increased production of CO_2 as well as higher water loss through diffusion across the eggshell *(17)*. While the ratio of CO_2 lost to O_2 gained through is between 0.70 – 0.75 in the avian egg, the O_2 molecule also has 27% lower mass, meaning that the mass exchanged due to the diffusion of these molecules is nearly equivalent (*17*). Therefore, during the course of incubation, the change in mass of the embryo can be attributed almost entirely to water loss. In our experiment we used the change in mass as an indirect measure of water loss. When we compared the water loss among eggs produced by control mothers, we saw an expected pattern. Embryos from juvenile control mothers lost 5.63×10^{-2} g (±0.474, ±95% CI) more water at a higher incubation temperature compared to the control incubation temperature (Fig. 1a) (Linear mixed model (LMM): $t = -2.47$, d.f. = 21, $P = 0.022$). However, mothers who were exposed to the mild heat conditioning as juveniles produced embryos that were more resistant to heat-associated water loss. Embryos from these heat-conditioned mothers did not lose more mass at higher incubation temperatures compared to those at a control temperature. (Fig. 1a) (LMM: $t = -0.223$, d.f. = 21, $P = 0.826$). In contrast, the thermal treatment mothers received as adults approximately three weeks before breeding had no effect on the change in egg mass during incubation. This effect on water loss cannot be attributed to differential egg size alone, as there were no differences in initial egg mass among the maternal treatment groups (LMM: juvenile: $t = -0.193$, d.f. = 37, $P = 0.848$; adult: $t = -0.406$, d.f. = 37, $P = 0.687$; J x A: $t = 0.280$, d.f. = 37, $P = 0.781$).

At standardized humidity, differences in embryonic water loss can be attributed in part to metabolic rate. Therefore, we investigated how maternal heat exposure shapes the metabolic rate of offspring as embryos. To do this, we measured embryonic heart rate – which is frequently used as a proxy for metabolic rate in birds and reptiles due to its strong correlation with oxygen consumption – at ~30% and ~77% of embryonic development (*30, 33*). As expected, there was a negative correlation between the embryonic heart rate and change in egg mass during development across all samples. For each 1 beat per minute (bpm) increase in heart rate, we saw a 4.92 $x10^{-4}$ g (±3.98 $x10^{-4}$, ±95% CI) increase in mass loss (LMM: t = -2.52, d.f. = 31, P = 0.017). However, this pattern did not hold true for all treatment groups. When incubated at the high temperature, embryos from mothers exposed to both heat treatments (Heat-Heat) had a significantly lower heart rates than embryos from all other maternal treatment groups at ~77% of embryonic development (Fig. 1b) (LMM: d.f. = 141, all P < 0.01).

Non-optimal incubation temperature can also result in changes of embryonic development time and/or differential hatchling morphology (*20, 29, 36, 37*). In such situations, embryos may prioritize development of essential organs such as the brain and heart at the expense of others (*17*). Here we saw an effect of the maternal juvenile treatment on embryonic development time, however this effect was dependent on the incubation temperature the embryos were exposed to. For embryos produced by juvenile control mothers, those that were incubated at the high temperature had a development time 0.435 days (±0.451, ±95% CI) shorter than those incubated at the control temperature as expected (Fig. 2a) (LMM: t = -1.94, d.f. = 47, P = 0.058). However, embryos produced by mothers exposed to the mild heat conditioning as juveniles had longer development times than the aforementioned embryos from juvenile control mothers, even when incubated at a high temperature (Fig. 2a) (LMM: HC-CH: β = 1.01, t = 2.89, d.f. = 47, P = 0.006; HH-CH: β = 0.947, t = 2.39, d.f. = 47, P = 0.021). Rather than being shorter, embryos produced by mothers that received the mild heat conditioning as juveniles had similar development times to those incubated at the control temperature (Fig. 2a) (LMM: CC-HC: β = 0.575, t = 1.88, d.f. = 47, P = 0.067; CC-HH: β = 0.512, t = 1.42, d.f. = 47, P = 0.162). These results again indicate that mothers exposed to heat as juveniles produced embryos that have altered physiological responses to high incubation temperatures that may be beneficial.

Across all samples there was a significant negative correlation between development time and embryonic heart rate at ~77% of embryonic development, a pattern similar to that demonstrated in other species (*38, 39*). For each 1 bpm increase in heart rate, we saw a 5.89 minute (±3.92 $x10^{-3}$, ±95% CI) decrease in embryonic development time (LMM: t = -2.10, d.f. = 46, P = 0.042). Interestingly, differential egg mass loss and heart rate patterns between treatment groups were also associated with morphology differences at hatching, but not in the manner we anticipated. Hatchlings produced by mothers who were exposed to the mild heat conditioning as juveniles had heavier pectoralis muscles when incubated at a high temperature. These hatchlings had pectoralis muscles 5.13 $x10^{-3}$ g (±4.57 $x10^{-3}$, ±95% CI) heavier than hatchlings produced by juvenile control mothers incubated at a control temperature (Fig. 2b) (LMM: pectoralis: t = 2.24, d.f. = 64, P = 0.029). Interestingly, this same group also exhibited a seemingly increased resistance to heat-induced water loss and development times unaffected by a higher

incubation temperature as previously described. These results suggest a potential adaptive maternal effect, as the increased pectoralis mass and elongated development times at high temperatures was not at the cost of reduced overall body mass or other measured organs (heart or residual yolk mass) (See Table S1 for full statistics). However, we cannot rule out that this change in embryo phenotype did not incur a cost that only becomes apparent later on in life. Phenotypic changes that promote increased fitness early on in life can come at the expense of longevity or reproductive output later in adulthood. Whether such trade-offs occur may also depend on the thermal environment the offspring would experience post-hatch. Phenotypic changes through maternal effects may only promote offspring fitness if the environment they experience is similar to that experienced by the mother, and detrimental if there is a mismatch.

Differential embryonic water loss may not only be attributed to metabolic rate, but also to eggshell traits. Lower pore density and thicker eggshells decrease gas conductance and therefore water loss. However, such morphology would also limit the rate of diffusion of oxygen and CO_2 across the eggshell, potentially decreasing the chance of survival at high temperatures. Therefore, we examined the role eggshell traits played in survival at different incubation temperatures. We found that successfully hatched individuals had 3.45 (±3.19, ±95% CI) more pores per cm^2 of eggshell compared to those that died (LMM: t = -2.16, d.f. = 75, P = 0.034) as well as eggshells that were 3.23 $x10^{-3}$ mm (±3.07 $x10^{-3}$, ±95% CI) thinner (LMM: t = 2.10, d.f. = 80, P = 0.039). We also saw that even among embryos that died, those with thinner eggshells and more pores tended to survive until further along in embryonic development (Fig. 3) (LMM: t = -2.42, P = 0.018). However, the positive correlation between the number of pores and survival time was only found in embryos exposed to the high incubation temperature (Fig. 3b) (LMM: t = 2.04, d.f. = 77, P = 0.0446), indicating the importance of eggshell characteristics in embryonic survival in this species at high ambient temperatures.

While it is clear that eggshell characteristics can influence the physiology, development, and survival of avian embryos, little is known about their plastic potential and ability to promote anticipatory maternal effects. Therefore, we examined how eggshell pore density and thickness are influenced by thermal conditions experienced by the mother. While we found no effect on eggshell thickness, we did find that mothers exposed to both heat treatments (Heat-Heat) laid eggs with 5.27 (±4.80, ±95% CI) more pores/cm^2 than Control-Control mothers (LMM: t = 2.24, d.f. = 29, P = 0.033). Interestingly, this increase in pore density did not correlate with increased water loss, even at high incubation temperatures. This may be due in part to the lower heart rates of embryos from Heat-Heat mothers at high temperatures, which paired with the increased pores potentially signals a reduced demand for oxygen rather than limitations in gas conductance.

In zebra finches, an incubation temperature of just one degree Celsius above optimum has been shown to decrease both hatching success and lean body mass in male embryos (*20, 29*). In this study, we saw an effect of maternal treatment on embryo survival, although not in the manner we anticipated. We presumed that the reduced heart rates and higher pore density shown by embryos produced by Heat-Heat mothers would promote increased survival at high temperatures, however this was not the case. Among

embryos exposed to the high incubation temperature, maternal treatment had no effect on survival (Fig. S2). However, at the control incubation temperature, embryos from mothers that experienced the mild heat as juveniles (Heat-Control) were 4.35 and 3.53 times as likely to survive and hatch successfully than those from mothers with "matching" treatments (Control-Control and Heat-Heat, respectively) (Fig. S2) (COXME: HC-CC: HR = 4.35, 95% CI = [0.469, 2.47], z = -2.88, P = 0.004; HC-HH: HR = 3.53, 95% CI = [0.383, 2.14], z = 2.81, P = 0.005). While maternal heat exposure resulted in physiological changes in embryos and survival at control incubation temperatures, it did not confer any benefits in survival in a "matching" environmental context, as expected to see in an anticipatory maternal effect. It is possible that the potential benefits or constraints of those physiological changes are only seen over a longer time-scale, and do not present themselves until later on in life.

While interpreting the adaptive significance of maternal effects is complex due to their highly context dependent nature, our study demonstrates the importance of designing experiments that incorporate several different time points and magnitudes of stressor exposure. Many of the effects on offspring phenotype we observed were the result of an interaction between maternal exposure to a mild and prolonged heat conditioning as juveniles, and high incubation temperatures experienced by their offspring. Such changes in offspring phenotype as the result of an interaction between "matching" maternal and offspring environment may signal the presence of anticipatory maternal effects, however, further research is required. Our findings support previous studies that have established early development as an important ontogenetic window for inducing transgenerational plasticity. The juvenile mild heat conditioning protocol used was not only effective at inducing maternal effects, but has also been previously shown to result in within-generation plasticity in this species (*22*). The duration of the mild heat conditioning likely played a significant role in its ability to generate both types of plasticity, as the 28 day treatment period represents a significant portion of the zebra finches developmental period. This finding supports the idea that environmental cues of a longer duration are more likely to induce anticipatory maternal effects, as they present a strong indicator that there will be high correlation between the maternal and offspring environment.

However, perhaps just as interesting was our finding that exposure to the high intensity heat stressor as adults did not induce a selfish maternal effect, or any effect independent of its interaction with the juvenile mild heat treatment. This is surprising, as the time just prior to and during a reproductive bout is considered another critical window for inducing maternal effects (*10*). Stressful environments can induce maternal effects at a cost to the mother, creating a position where mothers face a trade-off between self-maintenance and the current reproductive bout, often resulting in a decrease in offspring fitness (*3*). However, we observed no apparent detrimental effects of the maternal high heat stressor alone on the offspring or maternal phenotype (*see 22*). It is possible that trade-offs were avoided via plentiful resources, or the stressor was not strong enough or close enough to the reproductive bout to elicit a cost. Mothers had *ad lib* access to seed and water except when undergoing experimental treatments, potentially allowing them to increase food consumption to negate detrimental effects of the stressor. Often a longer environmental cue near reproduction will result in a stronger transgenerational effect (9*, 10, 40, 41*). If the high heat stressor mothers experienced as adults was a longer duration or closer to the

reproductive bout, we may have observed a more pronounced effect. Another possibility is that we did not capture the phenotypic costs because we did not measure the affected traits, or because they were not present at the time-scale at which we measured. Potentially, the cost of this stressor exposure may only be seen at a cellular level (*see 22*), that may only present at a macroscopic level later in life as reduced longevity (*42*). Similarly, we measured phenotypic traits in offspring only prenatally and immediately after hatch, and an induced selfish maternal effect may not be apparent until later on in development. Traits have different capacities for plasticity and involvement in trade-offs, which underpins the importance of measuring multiple traits and factoring in life-history in future studies on maternal effects moving forward. (*43*).

Our results suggest that thermal conditions experienced by the mother early on in life can act as a maternal effect, with the potential to increase embryonic survival, but not in an anticipatory manner as predicted. Such plasticity could play a key role in species that live in regions experiencing temperatures close to their upper thermal tolerance, which are thought to be at greater risk of diminished populations as a result of climate change. Our results also indicate that thermal conditions experienced by the mother, even very early on in life, elicits a phenotypic change in offspring prenatally. Such information is critical, as the embryos of many avian species develop near their upper thermal critical limits and have very little thermoregulatory capabilities own their own (*15, 16*). Even though avian parents can regulate egg temperature, their methods to prevent hyperthermia when ambient temperatures rise above the optimum incubation temperature often comes at a significant cost to themselves (*44*). As climate change causes increased temperatures worldwide as well as an increase in extreme temperature events, the importance of understanding the rescue capabilities of phenotypic plasticity for species grows (*2*). Understanding physiological and morphological adjustments of embryos due to maternal effects could improve our ability to predict population responses to climate change and pinpoint species that will be most at risk. Future studies in maternal effects would do well to utilize multiple ecologically relevant stressor magnitudes and exposure time points, as well as measuring a suite of traits in offspring over the course of their life-history in varying environments.

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Declarations

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Ethics statement

All the animal husbandry and experimental protocols found in this manuscript were approved by Auburn University's Institutional Animal Care and Use Committee (approval number: 2016–2826).

Statement of authorship

AJH and HW designed the study, AJH, LD, and HW carried out the experiment and collected data, AJH and HW performed statistical analyses and prepared the manuscript.

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Competing Interests

The authors declare no competing interests.

Supplementary Materials:

Methods – details on animal diet and husbandry and specific statistical models used

Table S1 and Figure S2

Eggshell data (.csv file)

Embryo and hatchling data (.csv file)

Figures

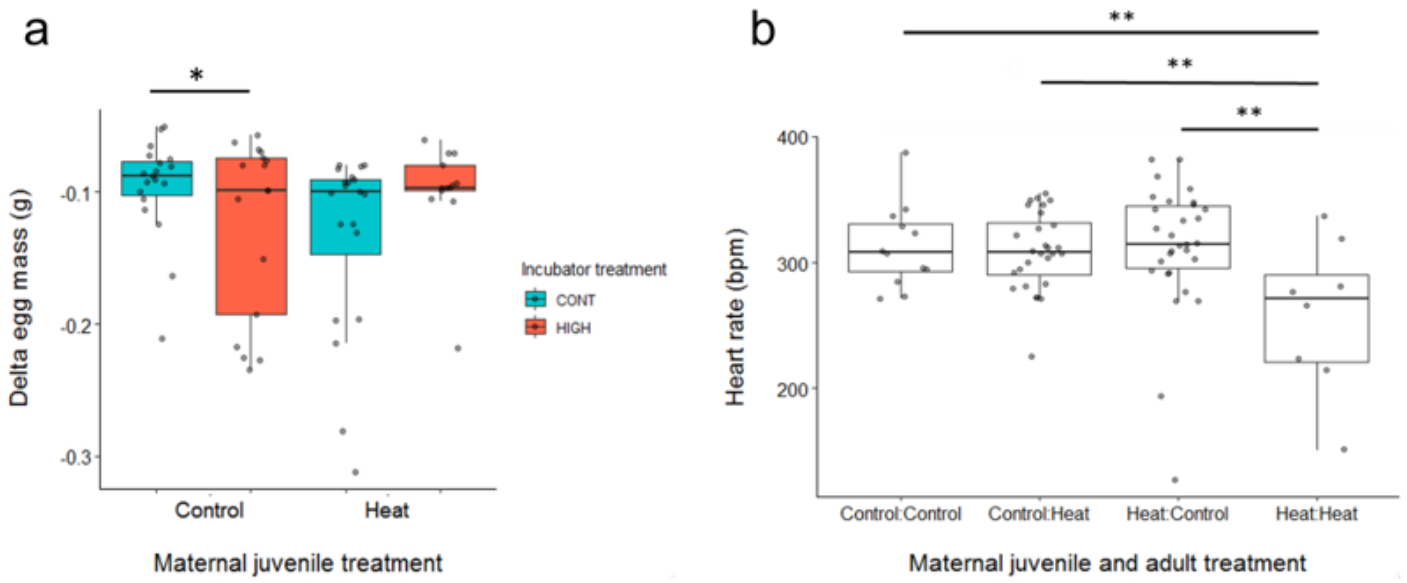

Figure 1

At high temperatures embryos showed differences in egg mass change and heart rate when produced by mothers exposed to a prior heat stress (significance indicated as follows for all graphs *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.). a. The change in egg mass (g) occurring from the day the egg was laid until approximately 77% of the incubation period. The x-axis denotes the treatment mothers received as juveniles, either a control (22°C) or mild heat (38°C) temperature for a prolonged period. The legend denotes the different incubation treatment groups that embryos produced by those mothers were exposed to, either a control (37.2°C) or high incubation temperature (38.5°C). **b**. The heart rates of embryos at the high incubation temperature (38.5°C) at approximately 77% of the incubation period. For each group on the x-axis, the first word denotes the treatment the mothers received as juveniles (control or

mild heat) and the second word denotes which treatment they received as adults (control or high heat). Boxplots show the median (horizontal line), lower quartile (median to end of box), upper quartile (median to top of box), minimums and maximums within 1.5 x interquartile range (whiskers) and outliers (data points beyond the whiskers).

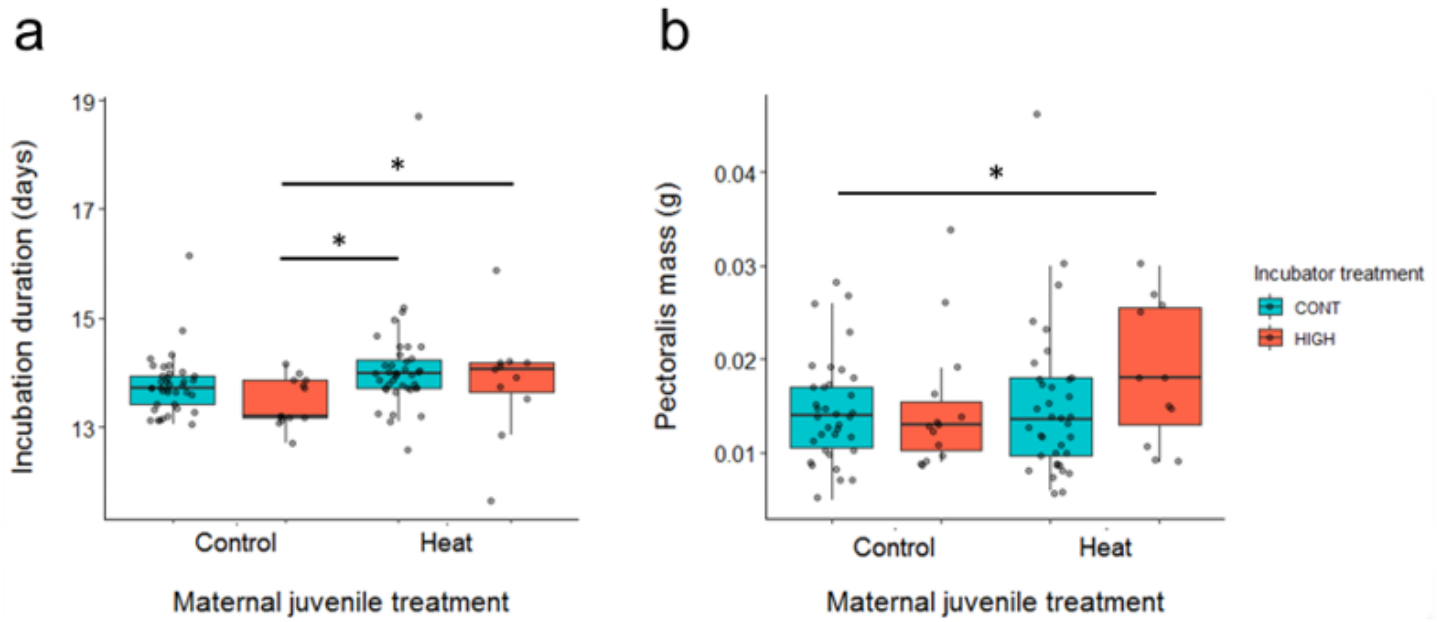

Figure 2

Embryos produced by mothers exposed to mild heat as juveniles had longer development durations and heavier pectoralis muscles at hatch. a. The total incubation duration (days) of embryos developed at different temperatures. The x-axis denotes the treatment mothers received as juveniles, either a control (22°C) or mild heat (38°C) temperature for a prolonged period. The legend denotes the different incubation treatment groups that embryos produced by those mothers were exposed to, either a control (37.2°C) or high incubation temperature (38.5°C). **b.** The total weight (g) of offspring pectoralis muscles after hatching. The x-axis denotes the treatment mothers received as juveniles, either a control (22°C) or mild heat (38°C) temperature for a prolonged period. The legend denotes the different incubation treatment groups that embryos produced by those mothers were exposed to, either a control (37.2°C) or high incubation temperature (38.5°C). Boxplots show the median (horizontal line), lower quartile (median to end of box), upper quartile (median to top of box), minimums and maximums within 1.5 x interquartile range (whiskers) and outliers (data points beyond the whiskers).

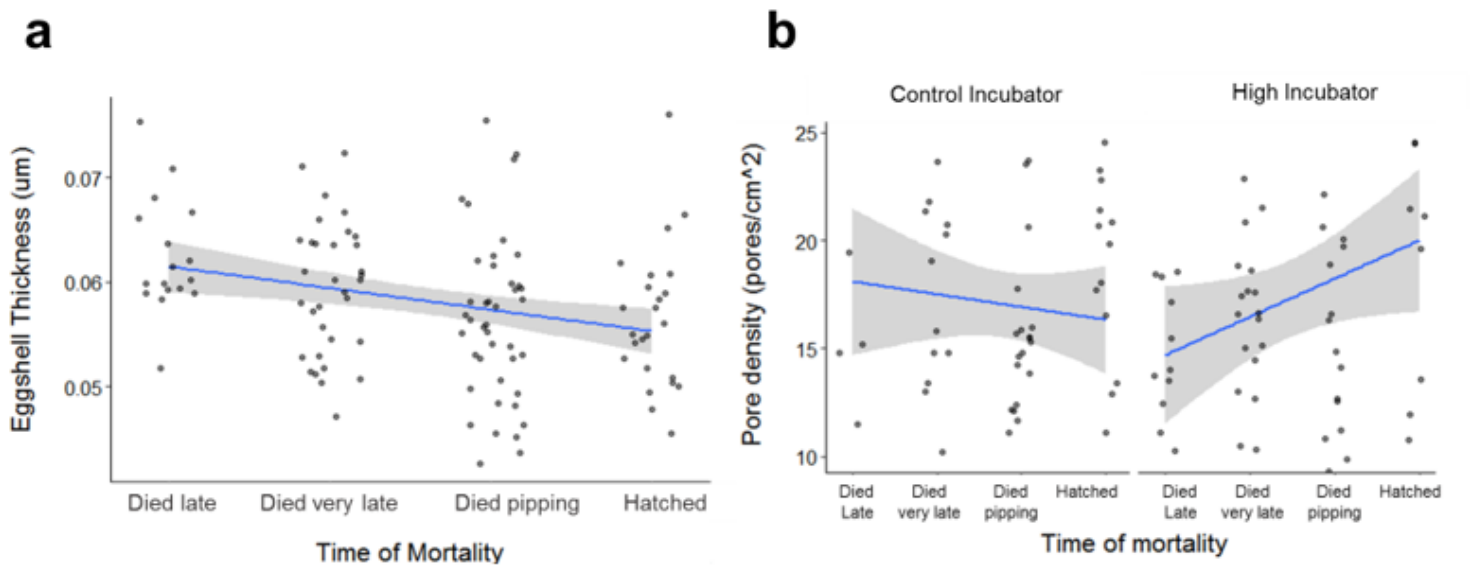

Figure 3

Embryonic survival in relation to eggshell thickness and pore density. For each graph the x-axis denotes the estimated developmental stage (very early, early, mid, late, very late, pipping) at which mortality occurred. **a.** The relationship between eggshell thickness (micrometers) and embryonic survival. **b.** The relationship between pore density (pores/cm^2) and embryonic survival at the two incubation temperatures (Control - 37.2°C, High - 38.5°C). The blue lines represent the regression line and shaded areas show 95% confidence intervals.

Supplementary Files

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- SupplementaryMaterialsFinalHoffmanetalv3.docx